

Purified human chorionic gonadotropin induces apoptosis in breast cancer

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Abstract

Agents that induce apoptosis in breast cancer cells have great potential to facilitate chemotherapeutic intervention and improve patient outcomes. In this study, the effects of injecting purified human chorionic gonadotropin (hCG) directly into human breast cancer xenografts grown in nude mice were examined. It was shown that intratumoral injection of purified hCG increased the apoptotic index in breast cancer xenografts. These results were supported by the findings that exposure of breast cancer cells to purified hCG decreased cell viability in five different breast cancer cell lines. In some of these cell lines, the effects of hCG in cell viability appear to correlate with activation/expression of the hCG/luteinizing hormone receptor. Preoperative apoptotic induction by factors such as purified hCG may improve local control or work synergistically with neoadjuvant chemotherapy to improve complete pathologic response of locally advanced breast cancer. [Mol Cancer Ther 2008;7(9):2837–44]

Introduction

Breast cancer is the most common cancer among women and the second leading cause of cancer deaths in women after lung cancer. The American Cancer Society estimates that more than 200,000 women are diagnosed with breast cancer each year in the United States alone. The outcomes of breast cancer treatment depend greatly on response to therapy and are predicted based on lymph node status and the extent of disease. Locally advanced breast cancers remain difficult to treat, and large tumors are less likely to be resected with negative margins. Although 75% to 95% of locally advanced breast cancers respond to neoadjuvant

chemotherapy, the pathologic complete response rate is less than 20%. Because patients with higher pathologic complete response rate have improved outcomes, identifying mechanisms to improve the pathologic complete response rate may affect survival in those with locally advanced breast cancers. Mechanisms that suppress apoptosis are suspected to be significant contributors to the development of intrinsic or acquired resistance to anti-cancer drugs and may prevent complete responses to neoadjuvant chemotherapy. Thus, the induction of apoptosis in breast cancer cells may facilitate therapeutic intervention and potentially improve outcomes.

A hormone that could be useful in the induction of apoptosis in breast cancer cells is human chorionic gonadotropin (hCG). It is well known that hCG belongs to both the glycoprotein hormone and the cysteine knot growth factor families (1, 2). The biologically active molecule of hCG consists of two noncovalently linked subunits, the free hCG- α and hCG- β , which are encoded by several independent genes (1, 2). The levels of hCG increase exponentially during the first trimester of pregnancy; this is followed by a rapid decline to low steady-state levels after that (1, 2). In addition to its pregnancy-maintaining actions, hCG causes differentiation of the breast glandular epithelium, which in turn results in (a) inhibition of cell proliferation, (b) increase in DNA repair capabilities of this tissue, and (c) decrease in the binding of carcinogen to the mammary cell DNA (3, 4). Most hCG actions are mediated by a G-protein-coupled receptor, which also binds luteinizing hormone (LH; refs. 5, 6). In fact, consistent with hCG function in breast tissue, hCG/LH receptors have been detected in normal breast epithelial cells, breast cancer tissues, and breast cancer cell lines (7–12). This finding is critical considering that it has been proposed that hCG may be useful in the prevention and/or treatment of breast carcinoma (10, 13–15). Interestingly, several studies have reported that full-term pregnancy at a young age has a perceived protective effect against the development of breast cancer (16–20).

The aim of this study was to determine whether intratumoral injection of purified hCG could increase the apoptotic index in breast cancer xenografts and to examine whether hCG alters the viability of different breast cancer cell lines.

Materials and Methods

Animals

Twelve 8-week-old athymic female nude mice were purchased from Harlan Industries. All experiments involving animals were carried out according to the regulations of the University of South Florida Institutional Animal Care and Use Committee. Mice were fed Tekland 22/5 rodent

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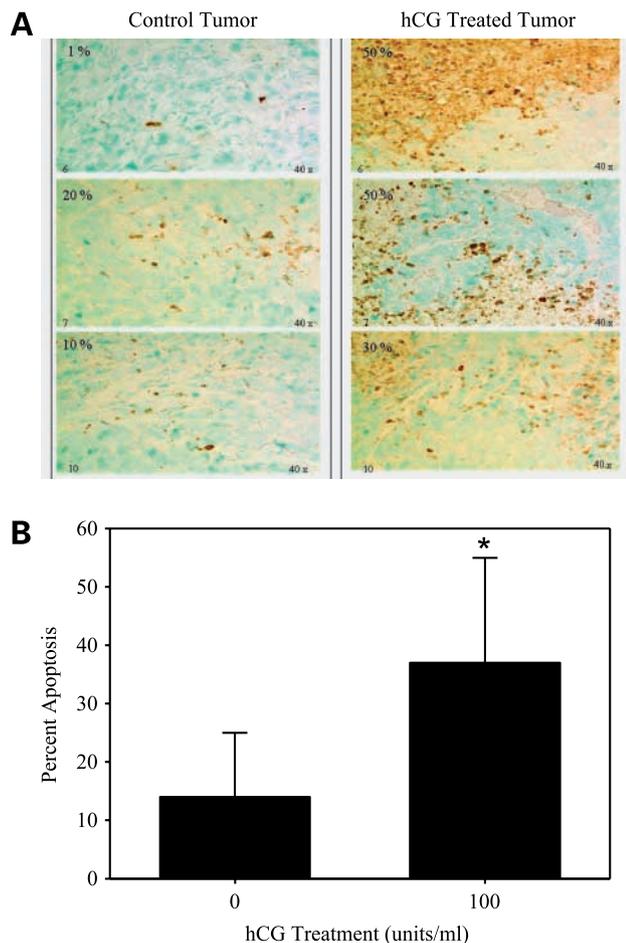


Figure 1. Treatment with hCG induces apoptosis in SKBR3 breast cancer xenografts. Tumor xenografts were grown in 8-wk-old athymic female nude mice as described in Materials and Methods. When tumors reached 150 mm³, 50 μ L of either 100 IU hCG or saline (control) was injected directly into matching flanks of tumor xenografts. Forty-eight hours later, the tumors were removed, fixed, and analyzed by the TUNEL assay. **A**, representative formalin-fixed sections of three matched pairs of SKBR3 xenografts tested by TUNEL assay. **B**, quantitated results for 12 pairs of tumor xenografts. Columns, mean; bars, SE. Treating of the tumor xenografts with hCG for 6 d resulted in almost complete necrosis of the tumor (data not shown). *, $P = 0.001$.

chow *ad libitum* and housed in a light-controlled room with 12 h of light followed by 12 h of darkness and had free access to water. To make the tumor xenografts, 5×10^6 SKBR3 cells were harvested, resuspended in PBS, and injected s.c. into the right and left flanks of the mice. When tumors reached 150 mm³, 50 μ L of 100 units nonrecombinant, semipurified hCG (A.P.L., Wyeth) was injected directly into the tumor xenografts of the left flanks, whereas the tumors in the right flanks received 50 μ L saline vehicle (control). After 48 h, tumors were harvested, snap frozen or fixed in 10% buffered formalin, and used in the different experiments.

Materials

The human breast cancer cell lines SKBR3, MCF7, MDA-MB-231, MDA-MB-468, and T47D were obtained from the

American Type Culture Collection. High-glucose and phenol red-free DMEM, gentamicin, 100 \times antibiotic-antimycotic, human estrogen receptor- α and - β (ER α and ER β , respectively)-specific antibodies, Vybrant MTT cell proliferation assay kit, and methyl green counterstain were purchased from Invitrogen. The ApoTag *in situ* apoptosis detection kit and the Chemicon mouse-to-mouse detection kit were from Chemicon International. The human Ki-67-specific antibody was purchased from BD PharMingen. DAB was obtained from DAKOCytomation. Radioimmuno-precipitation assay buffer, BCA protein assay kit, precast 4% to 20% SDS-PAGE, and SuperSignal West Pico chemiluminescent substrate were purchased from Pierce. Human LH receptor, progesterone receptor (PR), HER-1 (epidermal growth factor receptor), HER-2 (Neu), cyto-keratin 5/6, and β -actin-specific antibodies and the horse-radish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. All other chemicals were purchased from Fisher Scientific or Sigma-Aldrich.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay

DNA fragmentation in apoptotic cells was determined by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay with the ApoTag *in situ* apoptosis detection kit. For this, formalin-fixed, paraffin-embedded sections were cut at 4 to 5 μ m and air dried overnight at room temperature. Sections were then deparaffinized, rehydrated, and digested with proteinase K (25 μ g/mL in TBS) for 15 min using standard methods. After sections were quenched with 3% hydrogen peroxide, they were treated with equilibration buffer for 10 min. Incubation with terminal deoxynucleotidyl transferase in the presence of modified nucleotides was carried out for 60 min at 37°C, which resulted in the labeling of DNA fragments with the digoxigenin nucleotide. The reaction was stopped by incubating with stop-wash buffer for 10 min. On the DAKO autostainer, the digoxigenin nucleotide was incubated with anti-digoxigenin peroxidase (diluted 1:500) for 30 min and then with DAB chromogenic substrate for 10 min. Sections were manually counterstained with methyl green, dehydrated through graded alcohols, cleared in xylene, and mounted with resinous mounting medium. The evaluation of TUNEL was done at $\times 40$ magnification using an Olympus BX51 microscope. We scored the apoptotic cells as percent of total cells present in the field and evaluated three contiguous fields with >500 total cells present. The evaluation was done by two observers (D.C. and M.S.) who had no prior knowledge of the treatment. Discrepancy in interpretation was resolved by joint review by the two observers.

H&E Staining

Formalin-fixed, paraffin-embedded sections of tumor xenografts were cut at 3 μ m and allowed to air dry. Deparaffinized, rehydrated sections were stained for 6 min with Mayer's hematoxylin (modified AFIP recipe), washed in running tap water, and counterstained with eosin-phloxine (AFIP recipe). Sections were then dehydrated,

cleared with xylene, and mounted with resinous mounting medium.

Ki-67 Immunohistochemistry

Formalin-fixed, paraffin-embedded sections cut at 3 μ m were deparaffinized and rehydrated as described above. Sections received microwave antigen retrieval with 0.01 mol/L citrate buffer, pH 6.0 (high to boiling and then 20 min on power level 5; Emerson 1100 W microwave). After a 20 min cooling period, sections were rinsed with deionized water and placed in TBS-Tween 20 for 5 min. Slides were immunostained on a DAKO autostainer using the Chemicon mouse-to-mouse detection kit. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide. Slides were incubated with anti-human Ki-67 (dilution 1:400) for 30 min. DAB was used as the chromogen. After removal from the autostainer, slides were counterstained with modified Mayer's hematoxylin, dehydrated, cleared with xylene, and mounted with resinous mounting medium.

Tetrazolium-Based Colorimetric Assay (MTT)

All human breast cancer cells were maintained at a density of 10^8 per 75 cm³ flask in high-glucose DMEM supplemented with 10% fetal bovine serum, 1 \times antibiotic-antimycotic, and gentamicin. T47D and MCF7 cells were grown independently of estrogen. Cell viability was determined with the Vybrant MTT cell proliferation assay kit. For this, 1×10^4 breast cancer cells per well were seeded onto 96-well plates in 100 μ L culture medium. After 12 h of adherence, cells were treated with 100 units/mL (or as indicated) of purified hCG, 1 mmol/L (or as indicated)

of 8-bromo-cyclic AMP (8-Br-cAMP; Sigma-Aldrich) and/or 10 mmol/L protein kinase A inhibitor (PKI; Fisher Scientific) for the indicated time. After incubation with the different treatments, cells were exposed to the MTT dye (5 mg/mL) and incubated at 37°C for 3 h. The resulting formazan crystals were solubilized with DMSO, and the absorbance was measured at 540 nm with a multiscan autoreader (Dynatech MR 5000). The results are presented as mean ($n = 6$) \pm SE of percent viability relative to control. Some experiments were done in the presence of phenol red-free DMEM + 5% charcoal-stripped fetal bovine serum. In this case, cells were incubated in the presence of the experimental medium for 24 h before addition of hCG.

Western Blotting Analysis

Cell lysates were prepared from the indicated breast cancer cell lines using ice-cold radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors] and clarified by centrifugation. Protein concentrations of supernatants were determined by BCA protein assay kit (Pierce). Equivalent amounts of total cellular proteins were denatured at 100°C in loading buffer and subjected to electrophoresis on a precast 4% to 20% SDS-PAGE. After electrophoresis, samples were electroblotted onto nitrocellulose membranes (0.2 μ m pore) in buffer containing 25 mmol/L Tris-HCl (pH 8.3), 0.192 mol/L glycine, and 20% methanol for 1 h at 4°C. To verify equal protein loading, nitrocellulose membranes were stained with 0.1% Ponceau S (in 5% acetic acid) and destained in water. Western blot analysis for the different

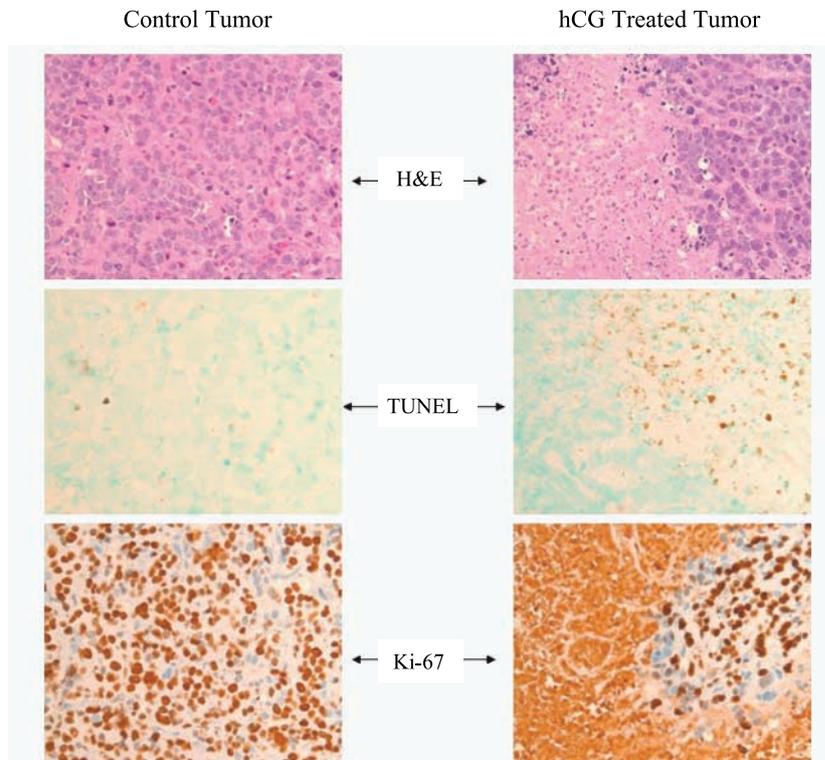


Figure 2. Ki-67 immunohistochemistry of a matched pair of SKBR3 breast cancer xenografts. Treatment of tumor xenografts with hCG or saline for 48 h was done as described in Fig. 1. H&E, TUNEL, and Ki-67 staining were obtained as described in Materials and Methods. Representative images for each type of staining in the tumor xenografts grown in the nude mouse 5 are shown.

proteins was carried out with 1:100 to 1:1,000 dilutions (depending on the antibody) of antibody specific for each protein in 5% milk/TBS-Tween 20. Immunoreactive proteins were visualized using a 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibody in 5% milk/TBS-Tween 20 and the SuperSignal West Pico chemiluminescent substrate (Pierce). Multiple exposures ranging from 5 s to 20 min were made.

Results and Discussion

To determine whether hCG could induce apoptosis in breast cancer xenografts, 50 μ L of either 100 IU semi-purified hCG or saline control was injected into matching flanks of SKBR3 breast cancer xenografts grown in nude mice. After 48 h, the tumors were harvested and analyzed by TUNEL assay as described in Materials and Methods. There was evidence of necrosis at the injection site, but clear apoptosis was detected in viable tissue by the TUNEL assay. Figure 1A illustrates the TUNEL assay results for three representative matched pairs. hCG-treated tumors

showed 37% apoptosis (range, 1-70%) within the xenografts compared with 14% apoptosis (range, 1-20%) observed in saline (control)-treated tumor xenografts ($P = 0.001$; Fig. 1B). Treating with hCG for 6 days resulted in almost complete necrosis of the tumor with evidence of apoptotic bodies (data not shown).

To examine whether this increase in apoptosis (Fig. 1) could be matched by an increase in cell proliferation, control and hCG-treated (48 hours) xenografts were tested by immunocytochemistry using a Ki-67-specific antibody. Representative images of H&E, TUNEL, and Ki-67 staining are depicted in Fig. 2. As shown, no significant differences were observed in cell proliferation as indicated by the Ki-67 staining patterns of saline (control) and hCG-treated xenografts. However, a significant increase in TUNEL staining was observed in hCG-treated xenografts compared with that shown in control samples. Similar results were obtained in other matched pairs (data not shown). These results suggest that direct, intratumoral injection of hCG can induce apoptosis in breast cancer cells without affecting cell proliferation. These data are in correlation

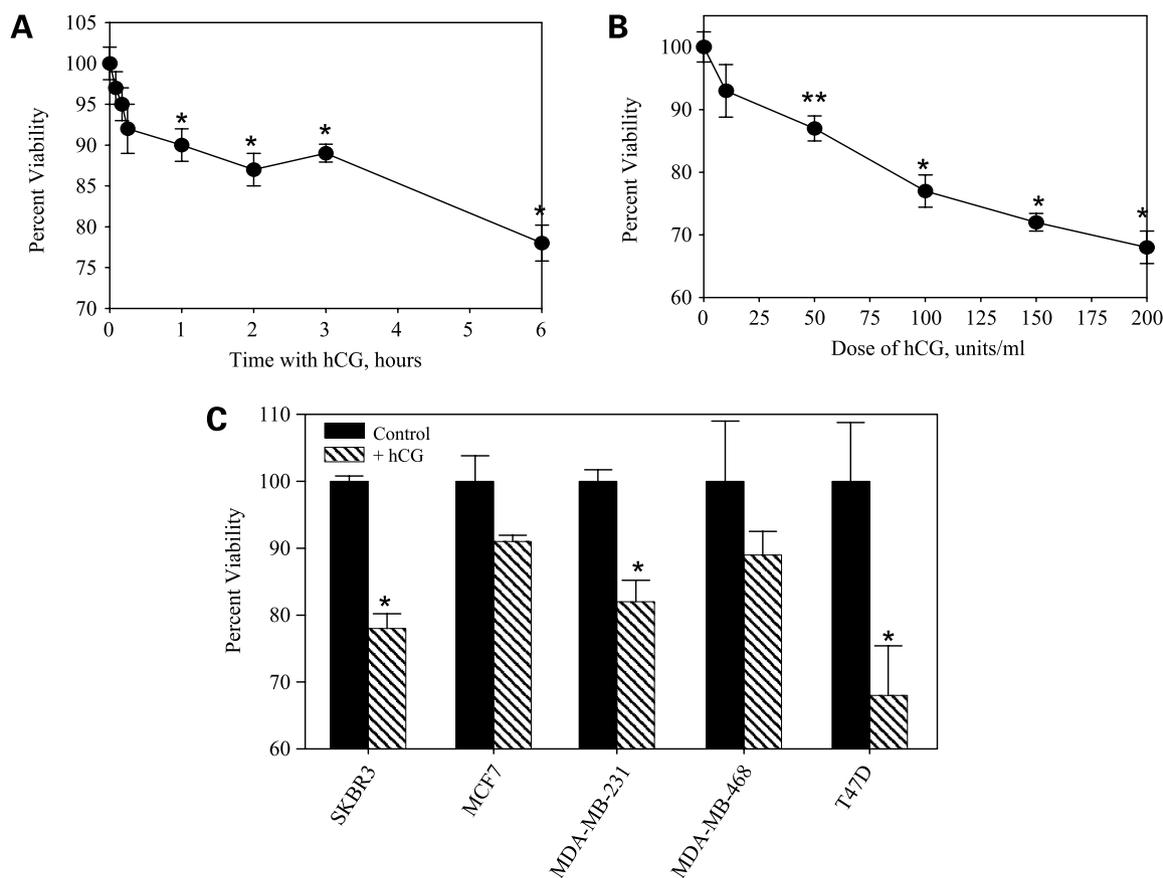


Figure 3. Effects of hCG treatment on breast cancer cell viability. Culturing and hCG treatment of the indicated breast cancer cell lines were done as described in Materials and Methods. Cell viability was detected using the MTT assay. Columns and data points, mean percent viability relative to the control ($n = 6$); bars, SE. **A**, time course for hCG treatment in SKBR3 cells. This experiment was repeated two times. *, $P < 0.001$. **B**, dose response for hCG in SKBR3 cells. Treatment with hCG was carried out for 6 d. *, $P < 0.001$; **, $P < 0.015$. **C**, effects of treatment with hCG for 6 d in five breast cancer cell lines. This experiment was repeated three times. *, $P < 0.001$.

with previous *in vivo* experiments indicating that hCG can inhibit the progression of 7,12-dimethylbenzanthracene-induced mammary carcinomas in rats through induction of apoptosis (21). Furthermore, hCG treatment induced an acceleration in the expression of apoptotic genes such as TRMP2, interleukin-1 β -converting enzyme, Bcl-X ς , c-Myc, and p53 or an up-regulation of the synthesis of inhibin, which is known to have tumor-suppressive activity (3, 4, 8, 21–24).

To confirm the results obtained with the xenografts, cell viability experiments were carried out *in vitro*. For this, SKBR3 cells were cultured in 96-well plates at a density of 1×10^4 per well and treated with hCG or vehicle control for the indicated times (Fig. 3A). MTT was carried out as described in Materials and Methods. As shown in Fig. 3A, the viability of the SKBR3 cells was reduced by hCG treatment in a time-dependent manner. Significant decreases in the viability of the SKBR3 cells were observed as soon as 24 h (10%; $P < 0.001$; Fig. 3A). The effects of hCG in SKBR3 cell viability also appeared to be dose dependent (Fig. 3B). Significant decreases in cell viability were observed with a dose as low as 50 units/mL (13%; $P = 0.015$; Fig. 3B). To further confirm the results, MCF7, MDA-MB-231, MDA-MB-468, and T47D breast cancer cells were also used in these experiments. In this case, hCG treatment was carried out for 6 days. As shown in Fig. 3C, cell viability was decreased in all of the cell lines tested. Significant decreases were observed in SKBR3 (22%; $P < 0.001$), MDA-MB-231 (18%; $P < 0.001$), and T47D (32%; $P < 0.001$) cells (Fig. 3C). Although the majority of these breast cancer cell lines also showed a time-dependent decrease in viability in response to hCG treatment, statistically significant decreases were observed typically at 6 days of treatment. The data show that hCG can decrease breast cancer cell viability and correlate with the increase in apoptosis observed in the xenograft experiments.

To investigate whether the reductions in cell viability in response to hCG could be influenced by the presence of other hormones in the medium, MTT experiments were carried out in the presence of phenol red-free medium supplemented with 5% charcoal-stripped serum. As shown in Fig. 4, the use of a hormone-deficient medium did not significantly alter the hCG-dependent reduction in the viability of SKBR3, MDA-MB-231, and T47D cells. Similar results were obtained in the case of MCF7 and MDA-MB-468 cells (data not shown).

In agreement with these findings is the report indicating that the culturing of MCF7 cells with hCG results in a hCG/LH receptor-dependent decrease in cell proliferation and invasion across Matrigel membranes (25). This is interesting considering that it has been shown that women with hCG/LH receptor-positive tumors have longer metastasis-free survival (10). Further confirmation of the effects of hCG in breast cancer was presented in pilot clinical studies that showed that hCG significantly reduced the proliferative index and the expression of both ER and PR in breast cancers independently of whether they were newly diagnosed or metastatic (13). Unlike the results

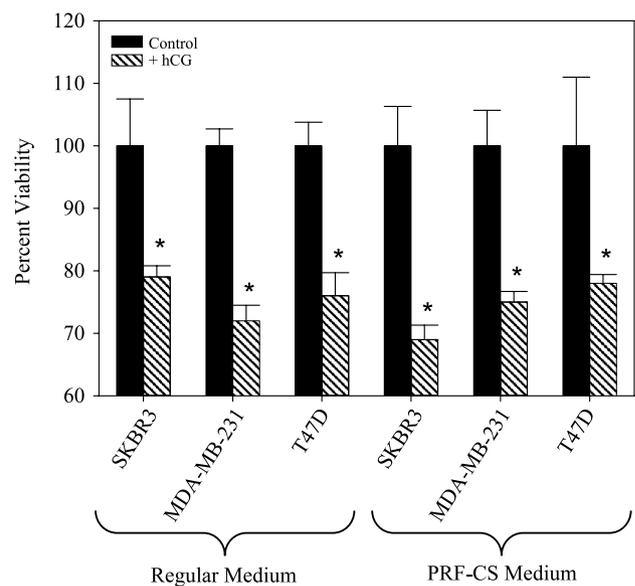


Figure 4. Influence of medium hormones on hCG-dependent reduction in the viability of breast cancer cells. Culturing of the cells in regular and hormone-deficient medium, treatment with hCG, and MTT assay were done as described in Fig. 3. Columns, mean percent viability relative to the control ($n = 6$); bars, SE. *, $P < 0.05$.

presented by Janssens et al. (13), we did not observe a decrease in the proliferative index of the SKBR3 xenografts. This discrepancy in response could be due to the use of different mode of administration (intramuscular versus intratumor), dosage (500 versus 20 μ g), and/or source of the hormone (recombinant versus purified) between the two studies. Additional examination is required to confirm these points.

The next step in our research was to determine whether the differences in hCG response observed in Fig. 3C were associated with hCG/LH receptor expression in these cell lines. As shown in Fig. 5, hCG/LH receptor expression was detected in MDA-MB-231 >> SKBR3 > T47D > MDA-MB-468 > MCF7. The hCG/LH receptor levels found in the MDA-MB-231 and SKBR3 cells corresponded with the significant response of these cell lines to hCG treatment. Interestingly, T47D showed the highest response in the MTT studies but had about 50% the amount of hCG/LH receptors found in the SKBR3 cells. Although MDA-MB-468 and MCF7 cells had detectable levels of hCG/LH receptor, their response in the MTT studies was relatively modest. Western blotting analysis was also carried out to identify markers expressed in each breast cancer cell line. As shown in Fig. 5, HER-1 was expressed in SKBR3 and MDA-MB-468 cells and at very low levels in T47D cells. HER-2 was detected in SKBR3 and at low levels in T47D cells, whereas ER α was expressed in MCF7 and T47D cells. T47D cells also expressed both isoforms of the PR (Fig. 5). None of these protein markers was detected in MDA-MB-231 (Fig. 5). No ER β or cytokeratin 5/6 was detected in any of the cell lines under analysis (Fig. 5).

One possible explanation for this lack of direct correlation between the response to hCG and the expression levels of hCG/LH receptors could be that the endogenous production of hCG in these cell lines masks the effects of the exogenously added hCG. In connection with this possibility, it has been reported that breast cancer cells are able to produce hCG (26). Interestingly, hCG- α is synthesized in high concentrations, especially in ER α -positive tumors (26), which could be associated with the low response of MCF7 to hCG treatment observed herein. The finding that T47D, which also expresses ER α , significantly responded to hCG treatment could be related to the presence of PR. PR has been shown to regulate the expression of hCG- β (27).

Finally, in an effort to corroborate the role of the hCG/LH receptor in the hCG-dependent reduction in breast cancer cell viability, MTT studies were carried out in the presence of cAMP, a downstream regulator of the hCG/LH receptor. MDA-MB-231 cells were selected for these studies due to their high levels of receptor expression (see Fig. 5). As illustrated in Fig. 6A, adding 1 mmol/L 8-Br-cAMP was sufficient to mimic the effects of hCG in these cells. As shown for hCG, the reduction in breast cancer cell viability by 8-Br-cAMP treatment was dose dependent (Fig. 6B). Treatment with PKI was able to block the response to both 8-Br-cAMP and hCG, showing the involvement of protein kinase A activation in this regulatory pathway (Fig. 6C). It is important to mention that this is not the first time that the role of cAMP in breast cancer has been investigated (28, 29). One study confirmed that treating MCF7 cells with

dibutyryl-cAMP reduces cell proliferation, a process that involved protein kinase A activation (28), whereas in another study 8-Br-cAMP administration blocked the ability of MCF7 cells not only to proliferate but also to grow in an anchorage-independent manner (29).

Another tumor that has been reported to be influenced by hCG treatment through induction of apoptosis is Kaposi's sarcoma, the most common tumor found in patients with acquired immune deficiency syndrome (30–32). Purified hCG has been shown to increase apoptosis in Kaposi's sarcoma cells both *in vitro* and *in vivo* (30–32). Interestingly, when highly purified or recombinant hCG and the hCG subunits were used in the studies with Kaposi's sarcoma, no effect was seen (31–34). Furthermore, different sources of clinical-grade hCG preparations varied in their anti-Kaposi's sarcoma activity (4, 24, 31–35). Attempts to decipher this contradiction lead to the identification of a hCG-associated factor, which appears to be responsible for the apoptotic activity of the hCG preparations (33, 34). hCG-associated factor is present in several commercial preparations of hCG, with A.P.L. (Wyeth), the inducing agent in this study, having the most activity. This hCG-associated factor could be a peptide, an associated protein, or even a breakdown product of hCG that could be found in the urine of pregnant women (31, 33, 35–37). In fact, it is known that the β -subunit of hCG is susceptible to proteolytic cleavage *in vivo*, which can produce peptides of the size of hCG-associated factor (38). Other factors that could be found in commercial hCG

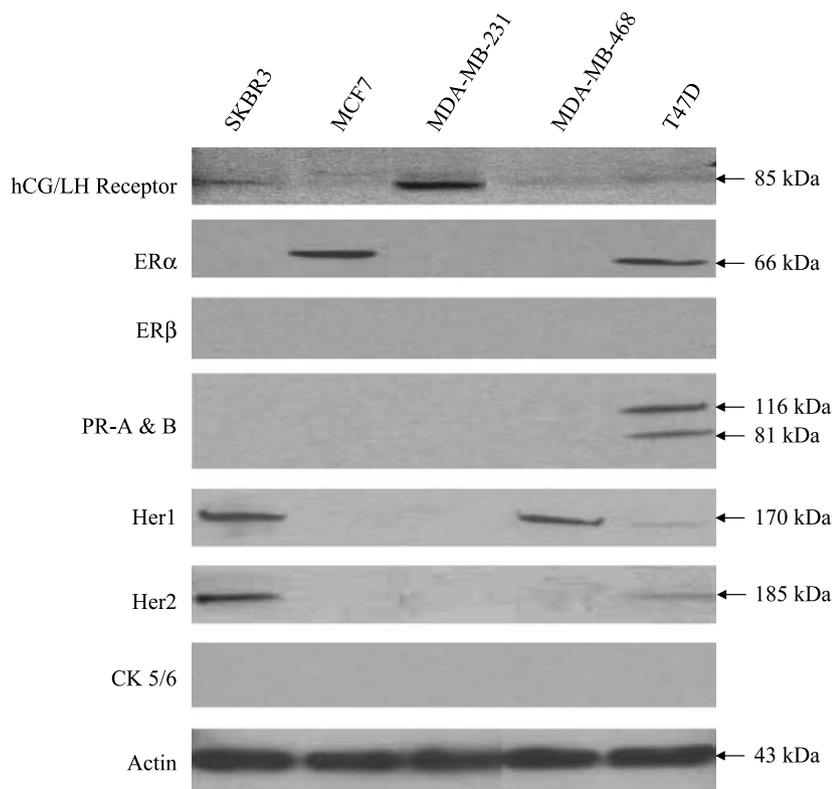


Figure 5. Expression of hCG/LH receptor, ER α , ER β , PR, HER-1, HER-2, and cytoke-
 ratin 5/6 (CK5/6) in the different breast cancer cell lines. Cellular extracts were prepared from the indicated breast cancer cell lines and analyzed by Western blotting analysis as described in Materials and Methods. Blots were probed with antibodies specific for the indicated proteins. Actin was used as the internal control. Representative Western blots are shown. *Right*, molecular weight for each protein.

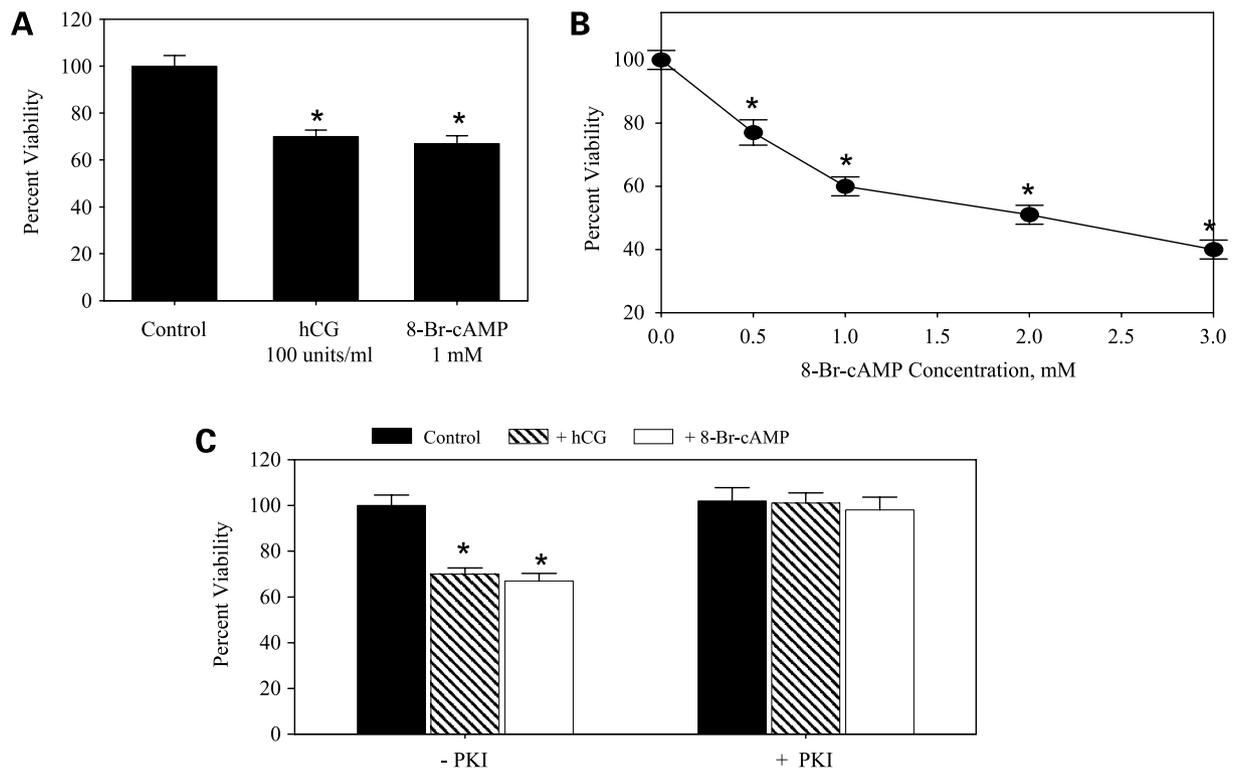


Figure 6. 8-Br-cAMP mimics the effects of hCG on breast cancer cell viability. MDA-MB-231 cells were used in these experiments. Cell viability was detected as described above. Columns and data points, mean percent viability relative to the control ($n = 6$); bars, SE. **A**, comparison between hCG and 8-Br-cAMP effects on cell viability. *, $P < 0.001$. **B**, dose response for 8-Br-cAMP treatment in MDA-MB-231 cells. *, $P < 0.001$. **C**, adding PKI prevented 8-Br-cAMP and hCG effects on cell viability. *, $P < 0.001$.

in different proportions and have been shown to be toxic to Kaposi's sarcoma cells include lysozyme, low-molecular-weight contaminants, and the eosinophil-derived neurotoxin RNase (31, 34, 35, 38). Although previous studies have shown that hCG itself has a direct effect in breast cancer (13, 25), additional experimentation is required to identify/purify hCG-associated factor and to determine its effects in breast cancer cells either alone or in conjunction with hCG. It is important to mention that, unlike breast cancer, Kaposi's sarcoma cells do not appear to express LH/hCG receptors (38).

In summary, we have identified a significant apoptotic induction in breast cancer xenografts after direct injection of purified hCG. Although further characterization of the exact mechanism(s) involved in this regulatory process is necessary, these experiments suggest a potential therapeutic advantage by intralesional injection to induce apoptosis in locally advanced breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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